AD_____

Award Number: DAMD17-01-1-0716

TITLE: Growth Mechanisms of Schwann Cell Tumors in NF2

PRINCIPAL INVESTIGATOR: Anat O. Stemmer-Rachamimov, M.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, MA 02114-2554

REPORT DATE: July 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Annual (1 Jul 2003 - 30 Jun 2004)

4. TITLE AND SUBTITLE
Growth Mechanisms of Schwann Cell Tumors in NF2

5. FUNDING NUMBERS
DAMD17-01-1-0716

6. AUTHOR(S)
Anat O. Stemmer-Rachamimov, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
Boston, MA 02114-2554

E-Mail: astemmerrachamimov@partners.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Patients with Neurofibromatosis 2 (NF2) develop multiple schwannomas that can cause significant morbidity and mortality and numerous, small, neoplastic Schwann cell tumorlets in the cauda equina that do not grow and are clinically silent[3-6]. Since *NF2* gene inactivation has been shown to occur in both tumorlets and schwannomas, our hypothesis is that additional genetic or epigenetic events are required for the development of frank, symptomatic schwannomas[7-11].

The purpose of this study is to identify with oligonucleotide expression microarray analysis, the growth-associated factors that drive the growth of symptomatic schwannomas. We collected and histologically examined tissues from 4 NF2 autopsies and identified specimens as schwannomas, tumorlets and normal peripheral nerves. RNA was extracted, amplified and checked from representative samples from each autopsy. The two autopsies with the superior RNA quality (as judged by Agilent Bioanalyzer system) were used for the microarray study. Laser captured tissues (to ensure the exclusion of adjacent normal tissues) of 7 schwannomas, 8 tumorlets and 2 nerves from these 2 autopsies were hybridized on Affymetrix U133 chips. Lists of differentially expressed genes in the tumorlets and schwannomas were compiled using multiple software programs and multiple analysis methods. Validation by RT PCR and immunohistochemistry is ongoing.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Neurofibromatosis 2, schwannoma, tumorlets, growth factors	8
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Patients with Neurofibromatosis 2 (NF2) are affected by multiple schwannomas that can cause significant morbidity and mortality. In addition to the schwannomas, NF2 patients also develop numerous, small, neoplastic Schwann cell tumorlets in the cauda equina that do not grow and are clinically silent[3-6]. Since have the *NF2* gene has been shown to be inactivated in both tumorlets and schwannomas, we have posed the hypothesis that additional genetic or epigenetic events are required for frank, symptomatic schwannomas to develop[7-11]. The purpose of this study is to find the specific growth-associated factors that drive the progression of some Schwann cell tumorlets to become symptomatic schwannomas, by using oligonucleotide expression microarray analysis.

PROGRESS REPORT BODY:

a) Approved Statement of Work:

Task 1: To compare the expression profile of NF2-associated Schwann cell tumorlets and frank schwannomas using microarray analysis and, if necessary, cDNA representational differential analysis (Months 1-10):

a. Laser-capture microdissection of Schwann cell tumorlets [10 samples] and schwannomas [10], RNA extraction and generation of cDNA.

b. microarray hybridization and analysis of data, statistical analysis and priorization.

c. cDNA-representational difference analysis on pairs of Schwann cell tumorlets and schwannomas [3 pairs].

Task 2: To evaluate and validate the candidate growth-promoting factors at the RNA and protein levels on NF2-associated schwannomas relative to tumorlets; NF2-associated schwannomas relative to tumor growth rate; sporadic schwannomas relative to sporadic Schwann cell tumorlets; and schwannomas relative to Schwann cell hyperplasia in murine NF2 models (months 11-36).

a. Northern blotting and RT-PCR, frozen tissues: Schwann cell tumorlets (NF2-associated [10 samples] and murine hyperplasia [10]) and schwannomas (NF2-associated [20], sporadic [30] and murine) [10].

b. Optimization of commercially available antibodies to candidate proteins

c. Generation of monoclonal and polyclonal antibodies to candidate proteins

d. Optimization of newly generated antibodies

e. Western blotting, frozen tissues: Schwann cell tumorlets (NF2-associated [10 samples] and murine hyperplasia [10]) and schwannomas (NF2-associated [20], sporadic [30] and murine) [10]

f. Immunohistochemical analysis, frozen and archival tissues: NF2-associated Schwann cell tumorlets [10 samples], NF2-associated schwannomas [70], sporadic tumorlets [5], sporadic schwannomas [30], murine hyperplasia [10] and murine schwannomas [10].

Task 3: To begin investigation of the mechanisms underlying overexpression of specific differentially expressed, growth-associated molecules (e.g., evaluation of gene amplification, cloning of promoter regions).

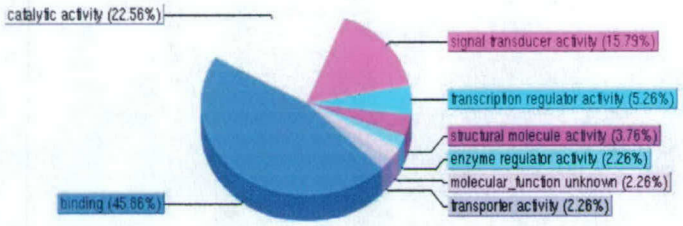
b.) Studies and Results

Task 1: Because NF2-associated Schwann cell tumorlets are clinically silent, these lesions can only be collected from autopsies. We have collected schwannomas, Schwann cell tumorlets and unaffected nerves from 5 NF2 patients that underwent autopsies. This large collection of tissues ensures sufficient number of "test tissues", i.e. analysis of tissues not included in the original class definition study, to be used for validation by immunohistochemical study of gene products identified by the microarray data.
All specimens were evaluated histologically (frozen sections) for histological verification of the lesion type. This was felt to be necessary because tumorlets, due to their small size, may be indistinguishable grossly from folds or kinks in normal nerves, and schwannomas may be indistinguishable from meningiomas by gross appearance alone (both tumor types are common in NF2 patients). This type of careful histological examination of each lesion, although time consuming and tedious, guaranteed that the micro-array profiling was performed on a homogeneous, well-characterized set of schwannomas and tumorlets. Selected samples of each of the NF2 autopsies were used for RNA extraction and amplification. The two autopsies with best quality of total RNA (by Agilent 2100 Bioanalyzer system) were used for the microarray analysis, while the other 3 cases will be used for immunohistochemical studies. As more cases become available, the information collected from the microarray and immunohistochemical analyses may allow some clinical correlations. For example, the expression of certain growth factors in tumors from a certain patient may correlate to total tumor load or to the clinical course.

In order to ascertain that the RNA is derived from the lesions alone and not contaminated by adjacent normal nerves, all lesions were laser captured from frozen sections. Although this is required only for the tumorlets that are deeply embedded in nerves, we chose to laser capture all the lesions to assure a uniform, standardized procedure, and avoid the possible introduction of a procedural bias by treating tumorlets and schwannomas differently. Therefor, all tissues (schwannomas, tumorlets, peripheral nerves) selected for microarray-analysis have been laser captured and the RNA extracted and amplified using the same protocols. RNA was extracted using the Picopure RNA Isolation kit (Arcturus Engineering) and two-round amplification was performed using the Arcturus Ribo-Amp RNA Amplification Kit (Arcturus Engineering). Amplified RNA was labeled with biotinylated uracil for the Affymetrix cDNA microarray chip (U133) using the ENZO High-Yield RNA Transcript Labeling Kit Protoco (Life Sciences). All samples were done in duplicates because Affymetrix's algorithm of data analysis software (MAS 5.0) works best if each data point consists of duplicated and the software can then provide p-values based on well-accepted statistical procedures.

Samples were then hybridized to the cDNA Affymetrix Human Genome U133 chip that screens 39,000 transcripts. The data is loaded into our Bio Informatics Programs of Affymetrix (MAS 5.0). This program was used to average the values for each transcript for each group and calculate the mean, standard deviation and standard error for each measurement. The program includes various analytical tools such as cluster analysis, Class Prediction paradigm and a variety of search tools that detect genes that behave in similar fashions across groups. Raw expression values were normalized by linear scaling, so that the mean array intensity was similar on all scans. Intensity thresholds were set at 20 and 20,000 units, resulting in 20160 transcripts for analysis. This probe set was subjected to t-test analysis using Vector Xpression and GeneSifter software for the identification of differentially expressed transcripts. Transcripts with a *P* value less than 0.01 were selected, yielding 88 up-regulated and 28 down-regulated genes. The breakdown of all differentially expressed genes by molecular function is shown in the figure bellow.

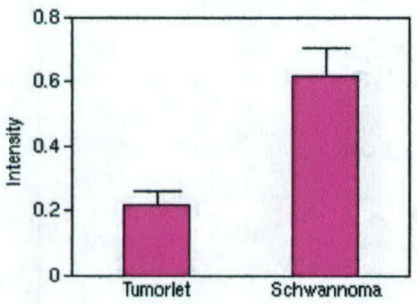
Figure 1: molecular function of differentially expressed genes



In addition, the 20160 probes were also analyzed with Gene Cluster, using the signal to noise metric. Overlapping the genes identified by these two separate methods yielded 14 up-regulated genes.

Of particular interest were some of the genes involved in signal transduction or in anti-apoptotic activity, for example anti-apoptotic gene shown in figure 2.

Figure 2



Task 2:

Prioritization of the up-regulated genes has been complete and data validation is being performed on the selected genes that showed the most differential expression. Some of the amplified RNA from each sample will be used for confirmatory analysis (in 5 selected genes, based on microarray gene expression data) using the Real Time Quantitative PCR (Taqman, ABI Prism 7700 System). This system monitors PCR at every cycle by

using a fluorogenic probe. It provides amplification plots of the sample as well as of standards (serial dilutions with known starting copy number). Therefor providing an evaluation of the copy number of the sample from the amplification plot (based on the threshold cycle that its plot crosses a defined fluorescence threshold). This is a very sensitive and reproducible technique, and provides confirmation to microarray expression data. We are using the pre-designed primer and probe sets now available from Applied Biosystems (2000 of the genes in the Affymetrix chip).

In addition, confirmation by immunohistochemistry will be performed. We are currently optimizing conditions for immunohistochemistry on paraffin embedded tissues for selected candidate gene products.

Task 3:

H&E sections of formalin-fixed, paraffin-embedded sporadic and NF2-associated schwannomas were reviewed for histological confirmation. Appropriate, representative blocks were selected and unstained sections were cut, to be used in the future for immunostaining for growth factors identified by the GeneChip analysis. Some of the tumors (50 cases) were also evaluated for nerve entrapment by neurofilament immunostaining (tumor invasion into adjacent nerve) and for proliferation activity by MIB1 immunostaining. These histological features will be correlated to the expression of gene products identified by the Affymetrix GeneChip. Murine lesions have been reviewed and examples of schwannomas and Schwann cell hyperplasia were identified.

KEY RESEARCH ACCOMPLISHMENTS:

- Acquisition of large numbers of tumorlets, schwannomas and normal peripheral nerves from NF2 patients
- Development and optimization of protocols for the harvesting, processing, laser capturing, extraction and amplification of laser-captured RNA (completed).
- Histological verification of the tissues collected on frozen sections
- Laser capturing of specimens (completed).
- RNA extraction, amplification and labeling of all laser captured samples (completed)
- Selection of cases to be analyzed by Affymetrix GeneChip based on quality and quantity of RNA evaluated by Agilent Bioanalyzer (completed).
- Analysis of samples by GeneChip (completed)
- Bio Informatics analysis of the expression data generated by the GeneChip (completed).
- Histological examination of H&E stained slides, selection of appropriate blocks and cutting of unstained sections from 50 schwannomas to be used for validation, by immunohistochemistry, of identified gene products (completed).
- Validation by RT PCR and immunohistochemistry differentially expressed genes (ongoing).
- Analysis of mouse lesions with immunohistochemistry for differentially expressed genes in human lesions (ongoing).

REPORTABLE OUTCOMES

NONE

CONCLUSIONS

Microarray expression study conducted on Schwann cell tumorlets and Schwannomas yielded a total of 116 differentially expressed genes: 88 up-regulated genes and 28 down regulated genes. Many of the genes are known to have a function in Schwann cell development and differentiation, some have anti-apoptotic functions, some are involved in signal transduction. These results are currently being validated by RT-PCR and by immunohistochemistry. These results, although preliminary, are exciting and may contribute a great deal to our understanding of tumor growth in schwannomas in NF2 patients.

REFERENCES

1. Evans DGR, Huson SM, Donnai D, Neary W, Blair V, Newton V, Harris R. A clinical study of type 2 neurofibromatosis. Quart J Med 304:603-618, 1992.
2. MacCollin M. Neurofibromatosis 2: clinical aspects. Neurofibromatosis. (JM Friedman, DH Guttman, M. MacCollin and VM Riccardi, eds.) Baltimore: John Hopkins 1999.
3. Louis DN, Stemmer-Rachamimov AO, Wiestler OD. Neurofibromatosis type 2. World Health Organization Classification of Tumours of the Central Nervous System. (P. Kleihues ad W. K. Cavanee, eds.) Lyon: IARC/WHO, 2000.
4. Louis DN, Ramesh V, Gusella JF. Neuropathology and molecular genetics of neurofibromatosis 2 and related tumors. Brain Pathol 5:163-172, 1995.
5. Rubinstein LJ. The malformative central nervous system lesions in the central and peripheral forms of neurofibromatosis. A neuropathological study of 22 cases. Ann NY Acad Sci 486:14-29, 1986.
6. Adelman LS, Aronson SM. Intramedullary nerve fiber and Schwann cell proliferation within the spinal cord (schwannosis). Neurol 22:726-731, 1972.
7. MacCollin M, Stemmer-Rachamimo AO. Neurofibromatosis 2: associated tumors. Neurofibromatosis. (JM Friedman, DH Guttman, M. MacCollin and VM Riccardi, eds.) Baltimore: John Hopkins 1999.
8. Sainz J, Huynh DP, Figueroa K, Ragge NK, Baser ME, Pulst SM. Mutations of the neurofibromatosis type 2 gene and lack of the gene product in vestibular schwanomas. Hum Mol Genet 3:885-891, 1994.
9. Twist EC, Ruttledge MH, Rousseau M, Sanson M, Papi L, Merel P, Delattre O, Thomas G, Rouleau GA. The neurofibromatosis type 2 gene is inactivated in schwannomas. Hum Mol Genet 3: 147-151, 1994.
10. Stemmer-Rachamimov AO, Xu L, Gonzalez-Agosti C, Burwick J, Pinney D, Beauchamp R, Jacoby LB, Gusella JF, Ramesh V, Louis DN. Universal absence of merlin, but not other ERM family members, in schwannomas. Am J Pathol 152:1649-1654, 1997.
11. Stemmer-Rachamimov AO, Ino Y, Lim ZY, Jacoby LB, MacCollin M, Gusella JF, Ramesh V, Louis DN. Loss of the NF2 gene and merlin occur by the tumorlet stage of schwannoma development in neurofibromatosis 2. J Neuropathol Exp Neurol 57:1164-7, 1998.